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***Mycobacterium abscessus* D-alanyl-D-alanine dipeptidase induces the maturation of dendritic cells and promotes Th1-biased immunity**

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Running Title: MAB1843 activates the functions of dendritic cells

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ABSTRACT

Mycobacterium abscessus, a member of the group of non-tuberculous mycobacteria, has been identified as an emerging pulmonary pathogen in humans. However, little is known about the protective immune response of antigen-presenting cells, such as dendritic cells (DCs), which guard against *M. abscessus* infection. The *M. abscessus* gene *MAB1843* encodes D-alanyl-D-alanine dipeptidase, which catalyzes the hydrolysis of D-alanyl-D-alanine dipeptide. We investigated whether MAB1843 is able to interact with DCs to enhance the effectiveness of the host's immune response. MAB1843 was found to induce DC maturation via toll-like receptor 4 and its downstream signaling pathways, such as the mitogen-activated protein kinase and nuclear factor kappa B pathways. In addition, MAB1843-treated DCs stimulated the proliferation of T cells and promoted Th1 polarization. Our results indicate that MAB1843 could potentially regulate the immune response to *M. abscessus*, making it important in the development of an effective vaccine against this mycobacterium.

Keywords: Dendritic cells, Th1 polarization, MAPK, *Mycobacterium abscessus*, MAB1843

1 INTRODUCTION

2 *Mycobacterium abscessus* is the most well known rapidly growing mycobacterium
3 (RGM), existing in natural habitats such as soil and water (1, 2) and usually found in
4 intracellular compartments within an infected host. Infection by this mycobacterium can
5 occur in various parts of the host's body, regardless of their immunity. Approximately 80% of
6 pulmonary RGM infections in the USA are attributed to *M. abscessus* (3). Unfortunately, the
7 resistance of this species to antituberculosis drugs and antibiotics has become a major
8 obstacle to its treatment in infected patients (4-6).

9 Recent studies have revealed that antigen-presenting cell (APC)-mediated innate and
10 adaptive immunities play a pivotal role in combating *M. abscessus* infection (7-9). These
11 studies also pointed out the importance of research on the pathogenicity of *M. abscessus* and
12 relevant immunotherapies. In the *M. abscessus*-infected mouse model, the interferon-gamma
13 (IFN- γ) and tumor necrosis factor alpha (TNF- α) secreted by various immune cells play a key
14 role as regulators of the bacterial infection (10, 11). Furthermore, *M. abscessus* induces
15 dendritic cell (DC) and macrophage secretion of TNF- α , interleukin-6 (IL-6), and IL-12p70
16 via the toll-like receptor 2 (TLR2) and TLR4 pathways (7, 8).

17 DCs are well known as the most powerful APCs. In peripheral tissues, immature DCs
18 express low levels of costimulatory and major histocompatibility complex (MHC) molecules
19 and exhibit a high level of endocytosis (12, 13). When DCs recognize pathogens via pattern
20 recognition receptors, they start maturing, and in the process express high levels of
21 costimulatory and MHC molecules, and proinflammatory cytokines, and their endocytosis
22 capacity is downregulated. In addition, DC maturation occurs as the cells migrate towards the
23 lymph nodes where they present antigens to naïve T cells, which induces T cell activation and
24 proliferation. In this way, DCs act as the link between innate immunity and adaptive

immunity, and hence, they have gained much attention in cancer immunotherapy research (12-15).

Mycobacterial proteins, such as PE-RGRS, LprA, MAB2560, Rv0652, Rv0462, MAP1305, HspX, and HBHA, have been shown to induce DC maturation and T cell activation (8, 16-22). These proteins activate TLR2 or TLR4 signaling and can be used as adjuvants for DC maturation. We recently suggested MAB2560 as the first ligand isolated from *M. abscessus* that induces the activation of DCs via TLR4; however, little is known about how *M. abscessus*-derived ligands affect the immune system.

MAB1843, a 222-amino-acid D-alanyl-D-alanine dipeptidase (D-Ala-D-Ala dipeptidase; EC number: 3.4.13.22), is a hypothetical antigen of *M. abscessus*. However, the function of MAB1843 in *M. abscessus* and the manner in which it affects the immune system remain unknown.

In this study, we aimed to elucidate the effects of MAB1843 on the immune system, with a focus on the maturation of DCs as well as their endocytosis and cytokine-producing abilities. In particular, we examined whether MAB1843 affects DC maturation through TLRs and how the activated DCs affect T cells. Knowledge on these mechanisms could contribute to the development of vaccines against *M. abscessus*.

RESULTS

MAB1843 Induces Dendritic Cell Maturation

First, we inspected the purity and toxicity of the recombinant MAB1843 before testing its physiological effect on DCs. The purity of MAB1843 was confirmed by Coomassie Brilliant Blue staining and western blot analysis using anti-His antibody (Ab). The apparent molecular size of the purified protein was approximately 24 kDa (Fig. 1A). In addition, the toxicity of MAB1843 on DCs was tested using Annexin V/propidium iodide (PI) staining and analyzed by fluorescence-activated cell sorting. Compared with the effect of a high dose of lipopolysaccharide (LPS; 1000 ng/mL) as a positive control, MAB1843 was not cytotoxic to DCs (Fig. 1B). To exclude the effects of endotoxin contamination, we also checked for endotoxins in the purified MAB1843. Using the limulus amoebocyte lysate (LAL) endotoxin assay kit (Lonza, Basel, Switzerland), we found the endotoxin levels in the purified MAB1843 to be < 0.1 EU/mL (Fig. 1C), indicating that certain amounts of MAB1843 are not cytotoxic to DCs. Next, to test the physiological effect of MAB1843 on DC maturation, we analyzed the expression of surface molecules and the secretion of cytokines in MAB1843- or LPS-induced (positive control) DCs. MAB1843 enhanced the expression of surface molecules such as CD80, CD86, and MHC class I and II (Fig. 2A), and promoted the secretion of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Fig. 2B), but His-tagged protein itself did not affect to the production of cytokines in DCs (Supplementary Figure 1). Whereas LPS stimulated the production of both IL-12p70 (involved in Th1 polarization) and IL-10 (involved in Th2 polarization), MAB1843 specifically induced the secretion of IL-12p70 only (Fig. 2C), indicating that it may promote Th1 polarization. Furthermore, MAB1843 suppressed the capacity of dextran and LPS to induce endocytosis in

DCs, indicating that it can functionally provoke the maturation of DCs towards promoting Th1 polarization.

MAB1843 Induces DC Maturation via TLR4 Signaling

Toll-like receptors of immune cells, such as macrophages and DCs, act as initiators of innate immunity by recognizing various pathogen-associated molecular patterns (PAMPs) (23, 24). Apart from PAMPs, other *M. abscessus* antigens can activate DC maturation through TLR2 or TLR4 (7, 8). Thus, we tested whether MAB1843 can modulate DC maturation through TLRs as well. We analyzed the secretion of proinflammatory cytokines, such as TNF- α and IL-6, in wild type (WT), TLR2 knockout (KO), and TLR4 KO DCs treated with MAB1843, LPS (TLR4 agonist), Pam3CSK4 (TLR1/2 agonist), imiquimod (TLR7 agonist), ODN1826 (TLR9 agonist), or Poly I:C (TLR3 agonist). Whereas the secretion of TNF- α and IL-6 was observed in all three cell types stimulated by imiquimod, ODN1826, or Poly I:C, both cytokines were completely inhibited in Pam3CSK4-stimulated TLR2 KO DCs and in LPS-stimulated TLR4 KO DCs. The secretion of cytokines was completely blocked only in TLR4 KO DCs stimulated by MAB1843 or LPS (Fig. 3A), indicating that MAB1843 can modulate DC maturation through TLR4. As mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF- κ B) signaling are reportedly downstream mediators in TLR4 signaling (24), we investigated whether MAB1843 could enhance the activation of these two mediators. We measured the phosphorylation of MAPKs and the expression of NF- κ B inhibitor-alpha (I κ B α) as an upstream molecule of NF- κ B in the cytosol. MAB1843 promoted the activation of MAPKs and NF- κ B in DCs derived from WT- and TLR2-knockout mouse but not TLR4-knockout mouse (Fig. 3B and Supplementary Figure 2). In addition, to investigate MAB1843 how to activate TLR4 signaling, we measured the interaction between TLR4 and MAB1843 using the BLitz system (Supplementary Figure 3).

These results indicate that MAB1843 could induce the DC activation occurs through the activation of TLR4-mediated MAPKs and NF- κ B signaling by direct binding with TLR4.

MAB1843 Enhances CD4⁺ and CD8⁺ T Cell Proliferation Through DC Maturation

Activated DCs mature during their migration to the lymph nodes. Once mature, they present antigens to naïve T cells and induce T cell proliferation (12). To characterize the effect of MAB1843 on DC and T cell interactions, we conducted a syngeneic mixed lymphocyte reaction assay using OT-I T-cell receptor (TCR) transgenic CD8⁺ T cells and OT-II TCR transgenic CD4⁺ T cells (25). The carboxyfluorescein succinimidyl ester (CFSE)-conjugated ovalbumin (OVA)-specific CD8⁺ and CD4⁺ T cells were divided and co-cultured with DCs presenting OVA_{257–264} (Fig. 4A) or OVA_{323–339} (Fig. 4B), which were effectively activated by MAB1843 treatment and LPS stimulation. In addition, MAB1843 also potentiated the production of IFN- γ by the activated CD4⁺ T cells, but not that of IL-4 (Fig. 4C). Considering these results and the fact that MAB1843 stimulated the DC production of IL-12p70 (Fig. 1C), we propose that this effect could be used in immunotherapy to polarize naïve CD4⁺ and CD8⁺ T cells towards an IFN- γ -producing Th1 phenotype.

DISCUSSION

Several strains of non-tuberculous mycobacteria, such as *Mycobacterium xenopi* and *Mycobacterium kansasii*, have been known to cause pulmonary diseases (26, 27). Recent research has shown that *M. abscessus* induces an immune response through TLR2 and dectin-1 on host cells (7, 9), and MAB2560, the first purified antigen isolated from *M. abscessus*, activates DCs via TLR4 (8). It is essential to understand the relationship between *M. abscessus* and the immune system to develop a treatment strategy against infection by this species. Therefore, it was necessary to complete the research on how the immune system responds to *M. abscessus*-derived proteins. In this study, we investigated the effect of MAB1843, another purified antigen isolated from *M. abscessus*, on the immune system.

Here, we report that MAB1843 plays a role as an adjuvant to induce the secretion of proinflammatory cytokines and Th1 polarization via DC stimulation. Specifically, MAB1843 induced the maturation of DCs and the secretion of proinflammatory cytokines (viz., IL-12p70, IL-1 β , IL-6, and TNF- α) via TLR4, both actions of which are important in T cell activation.

It has been reported that the MAPK and NF- κ B pathways, activated by TLR signaling, are involved in the maturation of DCs and the secretion of proinflammatory cytokines (28, 29). In line with these studies, we found that MAB1843 induced the activation of MAPKs and NF- κ B in DCs, which is important since DC maturation and proinflammatory cytokine production play a key role in T cell polarization. Therefore, it is possible that MAB1843 enhances the production of IL-12 in DCs through the activation of MAPKs and NF- κ B, which in turn elicits the differentiation of CD4⁺ T cells into Th1 cells, which produce IFN- γ . In line with these suggestions, we found that MAB1843 does indeed elicit Th1 polarization and IFN- γ production in Th1 cells. In this way, MAB1843 might be the link

1 between the innate immune response by DCs and the adaptive immune response by Th1 cells
2 to bacterial infection.

3 Our findings suggest that MAB1843 activates DCs and generates a Th1 response, as
4 well as induces Th1 polarization by enhancing IL-12 production in DCs and boosting IFN- γ
5 production in T cells. However, further *in vivo* studies are necessary to completely verify the
6 immune response to *M. abscessus*.

7 In conclusion, MAB1843 is a potent protein that induces DC maturation and Th1-
8 mediated immune response. Knowledge on the mechanism of how MAB1843 modulates the
9 activity of DCs could contribute to the development of vaccines against *M. abscessus*. The
10 identification of mycobacterial antigens that could be used as adjuvants to induce the
11 activation of APCs may direct the development of immunotherapy in the future.

MATERIALS AND METHODS

Animals

Female 4–6-week-old C57BL/6 (H-2K^b and I-A^b) mice were purchased from Orient Bio (Daejeon, South Korea). TLR2 and TLR4 KO mice, as well as transgenic OT-I and OT-II mice, were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All of the animals were housed in a specific pathogen-free facility and treated in accordance with the animal care guidelines (Institutional Animal Care and Use Committee (IACUC) number: KU14046).

Reagents and Antibodies

Recombinant mouse granulocyte macrophage colony stimulating factor (rmGM-CSF) was purchased from BioLegend (San Diego, CA, USA). The Annexin V/PI apoptosis detection kit was purchased from BD Biosciences (East Rutherford, NJ, USA). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated Abs—namely, FITC anti-mouse CD11c Abs (Clone: N418), PE anti-mouse CD80 Abs (Clone: 16-10A1), PE anti-mouse CD86 Abs (Clone: GL-1), PE anti-mouse H-2Kd/H-2Dd Abs (Clone: 34-1-2S), PE anti-mouse I-A/I-E Abs (Clone: M5/114.15.2), FITC anti-mouse IFN- γ Abs (Clone: XMG1.2), and PE anti-mouse IL-4 Abs (Clone: BVD4-1D11)—were purchased from BioLegend. TNF- α , IL-1 β , IL-6, IL-10, and IL-12p70 ELISA kits were purchased from eBioscience (San Diego, CA, USA). FITC-conjugated dextran was purchased from Sigma-Aldrich (St. Louis, MO, USA). The TLR agonists (viz., LPS from *Escherichia coli* O111:B4, Pam3CSK4, imiquimod, Poly I:C, and ODN1826) were purchased from Invivogen (San Diego, CA, USA). Antibodies against phospho-ERK, phospho-p38, phospho-JNK, I κ B α , and β -actin were purchased from Santa

Cruz Biotechnology (Santa Cruz, CA, USA). CFSE was purchased from Life Technologies (Eugene, OR, USA).

Cloning of the *MAB1843* Gene

The *MAB1843* gene was isolated from the full genome of *M. abscessus* for cloning into the *E. coli* BL21 strain. *MAB1843* was amplified using a forward primer (5'-CATATG ATGAAGCGTCTGATCCTCGGT-3') and a reverse primer (5'-AAGCTT GTTGACGGGCGCGTTCAGGAA-3'). Thereafter, the PCR products were cleaved by NdeI and HindIII, and the cleaved genes were then inserted into the pET-22b(+) vector (Novagen, Madison, WI, USA).

Purification of *MAB1843* from *E. coli* BL21

The transformed *E. coli* BL21 was incubated in Luria-Bertani broth containing 100 µg/mL of ampicillin. When the optical density at 600 nm reached 0.4–0.6, 1 mM of isopropylthio-β-D-galactoside was added to the *E. coli* BL21 culture medium, which was then incubated for 6 h. The *E. coli* BL21 cells were then harvested and lysed using lysozyme, dithiothreitol, phenylmethanesulfonyl fluoride (PMSF), and DNase I. The supernatant was analyzed by affinity chromatography with Ni-NTA agarose resin. The eluted solution was concentrated and dialyzed against phosphate-buffered saline (PBS). Finally, endotoxins were removed by polymyxin B-agarose (Sigma-Aldrich), and the endotoxin level was measured using the LAL test kit (Lonza).

Generation of Murine Bone Marrow Dendritic Cells

DCs were collected from the bone marrow of the tibia and femora of C57BL/6 mice. The progenitor cells were isolated from the bone marrow, and red blood cell lysis buffer was

added (Sigma-Aldrich). These cells were then mixed with RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and GM-CSF. The cells were seeded into 12-well cell culture plates and incubated at 37°C in a 5% CO₂ atmosphere. Fresh medium was added on the third day. On the sixth day, the cells were further treated or harvested.

Western Blot Analysis

The harvested cells were washed with PBS and lysed using a lysis buffer (containing 0.5% NP-40, 1 mM EDTA, 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5 mM PMSF, 0.5 M NaF, and 0.01% protease inhibitor cocktail (Biobasic, Amherst, NY, USA)). SDS-PAGE was conducted using a 10% polyacrylamide gel, following which the protein bands were transferred to a polyvinylidene difluoride membrane. After blocking the membrane with 5% skimmed milk, primary Abs (anti-p-p38, anti-p-ERK, anti-p-JNK, anti-IκBα, and anti-β-actin) were added to the membrane and incubation was carried out overnight at 4°C. On the next day, after rinsing with Tris-buffered saline and Tween 20, the anti-mouse and anti-rabbit secondary Abs were added to the membrane and a further incubation for 1 h at room temperature (RT) was carried out. After rinsing the membrane, enhanced chemiluminescence solution was added, and target bands were identified using the LAS-4000 imaging system (Fuji Film, Tokyo, Japan). To enable interaction with other antibodies, stripping buffer (Thermo Scientific, Waltham, MA, USA) was added to the membrane, following which the blocking step was repeated, and new antibodies were added.

Cytotoxicity Analysis

MAB1843- or LPS-treated DCs were harvested and rinsed with PBS. Annexin V/PI staining was performed on these cells for 15 min at RT. The cytotoxicity of MAB1843 on DCs was analyzed by flow cytometry using the FACS Calibur instrument (BD Biosciences).

Endocytosis Ability Analysis

MAB1843- and LPS-treated DCs were treated with FITC-conjugated dextran and incubated for 45 min at 37°C and 4°C, respectively. The DCs were then harvested and rinsed with PBS. Thereafter, the DCs were stained using the PE-conjugated anti-CD11c Ab and analyzed by flow cytometry.

Mixed Lymphocyte Reaction (MLR)

MAB1843- or LPS-treated DCs were incubated with OVA_{257–264} or OVA_{323–339}. Then, 1×10^4 DCs and 1×10^5 CFSE-labeled T cells were co-cultured in a 96-well cell culture plate. These transgenic OVA-specific CD8⁺ and CD4⁺ T cells were isolated from splenocytes of OT-I and OT-II mice, respectively. After 96 h, the cells were harvested and rinsed with PBS. Then, the cells were stained with Cy5 anti-CD8 or Cy5 anti-CD4 Abs and further analyzed by flow cytometry.

Intracellular Staining

The harvested and rinsed cells were fixed in 4% paraformaldehyde and then permeabilized using saponin buffer. The cells were stained with anti-IFN- γ and anti-IL-4 Abs for 20 min.

Enzyme-Linked Immunosorbent Assay

The levels of IL-12p70, IL-10, IL-1 β , TNF- α , IL-6, and IFN- γ were measured by ELISA (eBioscience; R&D Systems, Minneapolis, MN, USA).

Blitz assay

The direct binding between rmTLR4/MD2 and MAB1843 was performed using the BLItz system (ForteBio, Menlo Park, CA). Human rTLR4/MD2 tagged with anti-penta-His (HIS) was purchased from R&D Systems (Minneapolis, MN). HIS biosensors (catalog no. 18-5078, Fortebio) were hydrated for 10 min prior to the experiment. The concentration of HIS-tagged rmTLR4/MD2 was 0.1 mg/ml, and that of purified MAB1843 was 0.1, 0.5, and 1 mg/mL and BSA was 1 mg/mL. The setting was as follows: initial baseline for 30 s, loading for 300 s, baseline for 60 s, association for 120 s, and dissociation for 120 s.

Statistical analysis

All experiments were repeated at least three times, with consistent results. Unless otherwise stated, data are expressed as the mean \pm SEM. Variance analysis was used to compare experimental groups with control values, whereas comparisons between multiple groups were made using Tukey's multiple comparison tests (Prism 3.0; GraphPad software). A *P* value of less than 0.05 was considered to indicate statistical significance.

Conflict of interests

All of the authors have no relevant conflict of interests.

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FIGURE LEGENDS

Fig. 1. Purification and cytotoxicity of recombinant MAB1843. (A) Recombinant MAB1843 was purified by affinity chromatography with Ni-NTA resin and then subjected to SDS-PAGE and staining with Coomassie Brilliant Blue (left panel), as well as analysis by western blotting using 1:1000 mouse anti-His tag antibodies (right panel). (B) Dendritic cells were treated with the indicated concentrations of MAB1843 and LPS for 24 h and then stained with Annexin V/PI. The results are representative of three independent experiments. (C) Endotoxin levels in the purified MAB1843 were analyzed by the LAL endotoxin assay kit.

Fig. 2. MAB1843 induces the maturation of dendritic cells (DCs). (A) Immature DCs were cultured with 0.5 or 1 $\mu\text{g/mL}$ of MAB1843 or 50 ng/mL of LPS for 24 h and analyzed for the expression of surface markers by two-color flow cytometry. DCs were stained with FITC anti-CD11c antibodies and PE anti-CD80 antibodies, PE anti-CD86 antibodies, PE anti-MHC class I antibodies, or PE anti-MHC class II antibodies. (B and C) Immature DCs were cultured with 0.5 or 1 $\mu\text{g/mL}$ of MAB1843 or 50 ng/mL of LPS for 24 h. The amounts of TNF- α , IL-1 β , IL-6, IL-12p70, and IL-10 in the culture medium were measured by ELISA. The experiments were run at least in triplicates. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, when compared with DCs treated with MAB1843 only. (D) Mature DCs were generated by stimulating the immature cells with 50 ng/mL of LPS or 0.5 or 1 $\mu\text{g/mL}$ of MAB1843 for 24 h. Endocytic activity at 37°C and at 4°C was assessed by flow cytometry, based on dextran-FITC uptake. The percentages of dextran-FITC⁺ CD11c⁺ cells are indicated. The results are representative of three independent experiments.

Fig. 3. MAB1843 induces TLR4 signaling during dendritic cell (DC) maturation. (A)

DCs derived from wild type (WT), TLR2 knockout (KO), and TLR4 KO mice were treated with MAB1843 (1 $\mu\text{g/mL}$), LPS (50 ng/mL), Pam3CSK4 (Pam3) (10 $\mu\text{g/mL}$), imiquimod (Imiq) (1 $\mu\text{g/mL}$), ODN1826 (ODN) (1 $\mu\text{g/mL}$), or Poly I:C (10 $\mu\text{g/mL}$) for 24 h. The production of TNF- α and IL-6 in DCs was measured by ELISA. The experiments were run at least in triplicates. *** $P < 0.001$. (B) DCs were treated with MAB1843 (1 $\mu\text{g/mL}$) or LPS (50 ng/mL) for 0, 5, 15, 30, and 60 min. Anti-p-p38 antibody, anti-p-ERK antibody, anti-p-JNK antibody, anti-p38 antibody, anti-ERK antibody, anti-JNK antibody, anti-I κ B α antibody, and anti- β -actin antibody was added to the western blot membrane. The results are representative of three independent experiments.

Fig. 4. MAB1843 induces T cell proliferation and Th1 polarization. (A and B) Transgenic

OVA-specific CD8⁺ T cells and transgenic OVA-specific CD4⁺ T cells were isolated from OT-I and OT-II mice, respectively. T cells were stained with carboxyfluorescein succinimidyl ester and co-cultured for 96 h with DCs treated with 1 $\mu\text{g/mL}$ of MAB1843 or 50 ng/mL of LPS. The cells were then analyzed by flow cytometry. (C) Transgenic OVA-specific CD4⁺ T cells were co-cultured for 3 days with non-stimulated DCs, OVA₃₂₃₋₃₃₉-pulsed DCs, OVA₃₂₃₋₃₃₉-pulsed MAB1843-DCs, or OVA₃₂₃₋₃₃₉-pulsed LPS-treated DCs. The CD4⁺ T cell expression of IFN- γ and IL-4 was detected by intracellular staining and flow cytometry. The results are representative of three independent experiments.

Figure 1

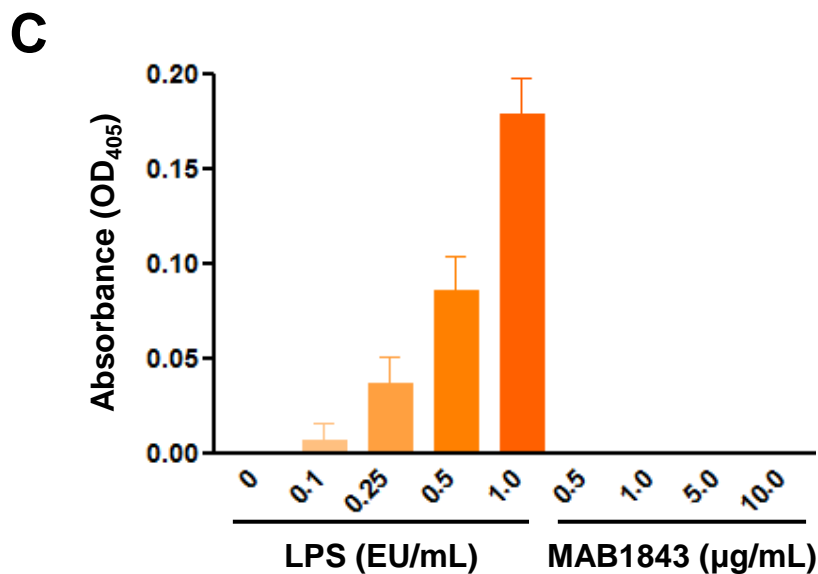
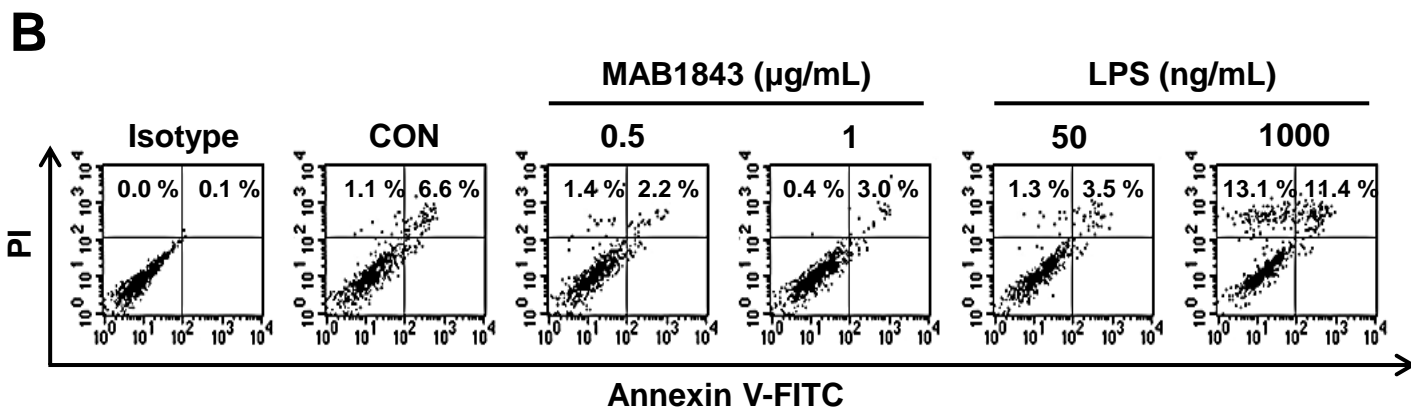
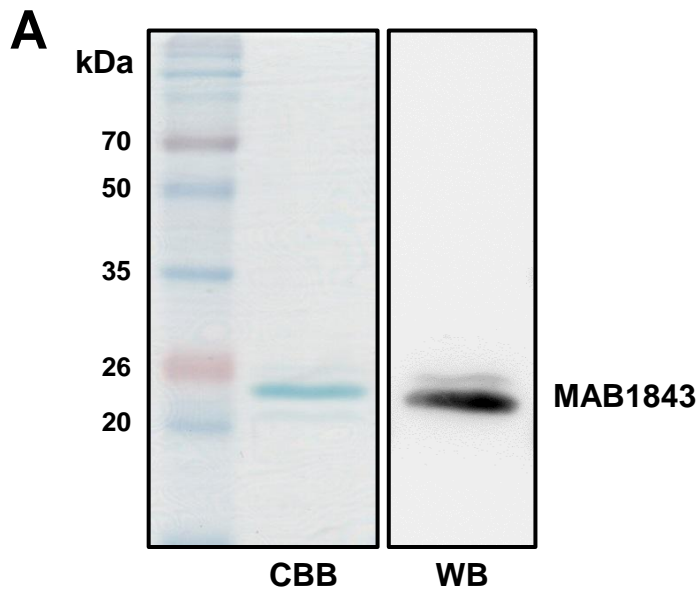
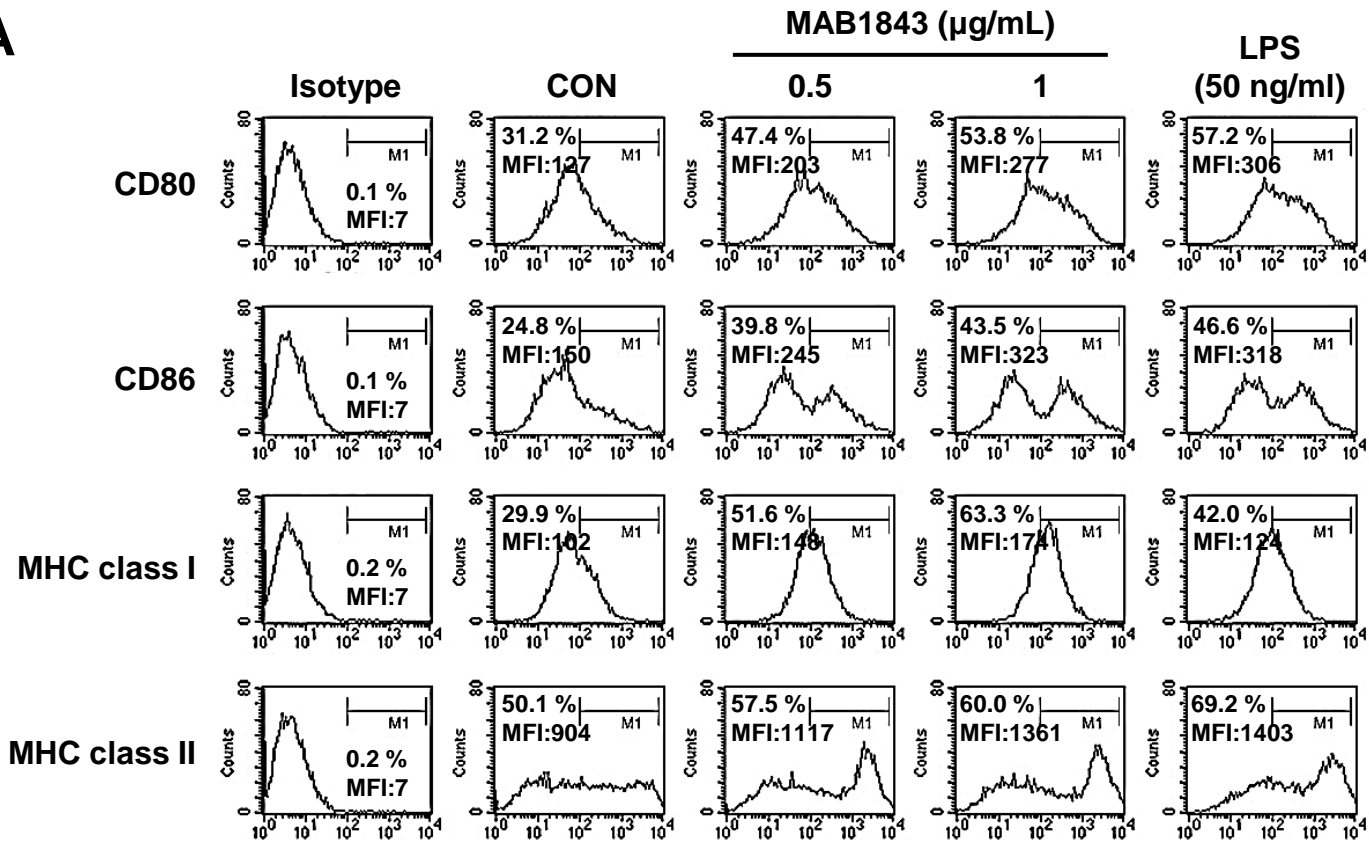
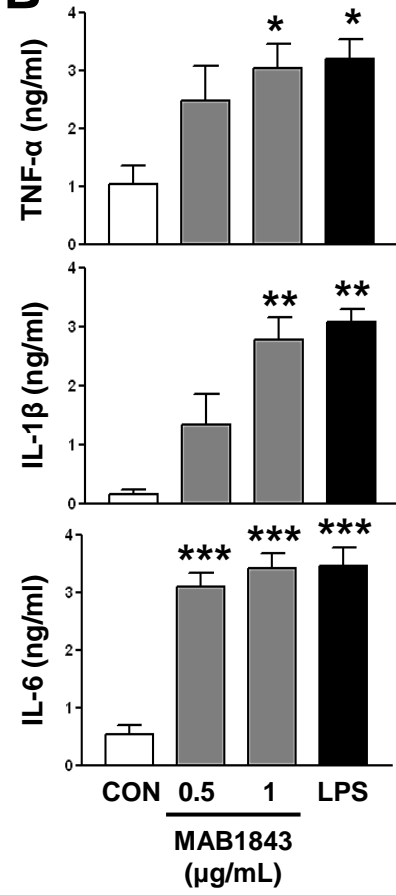


Figure 2

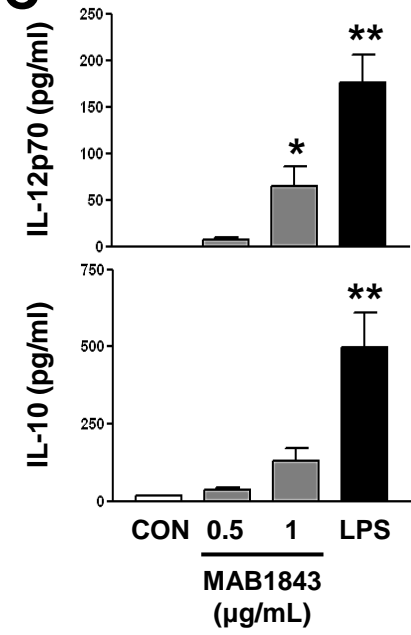
A



B



C



D

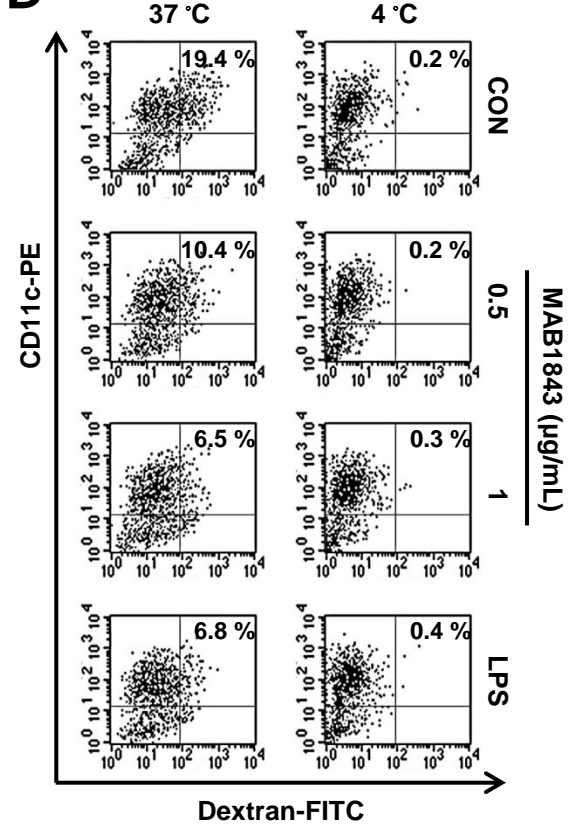


Figure 3

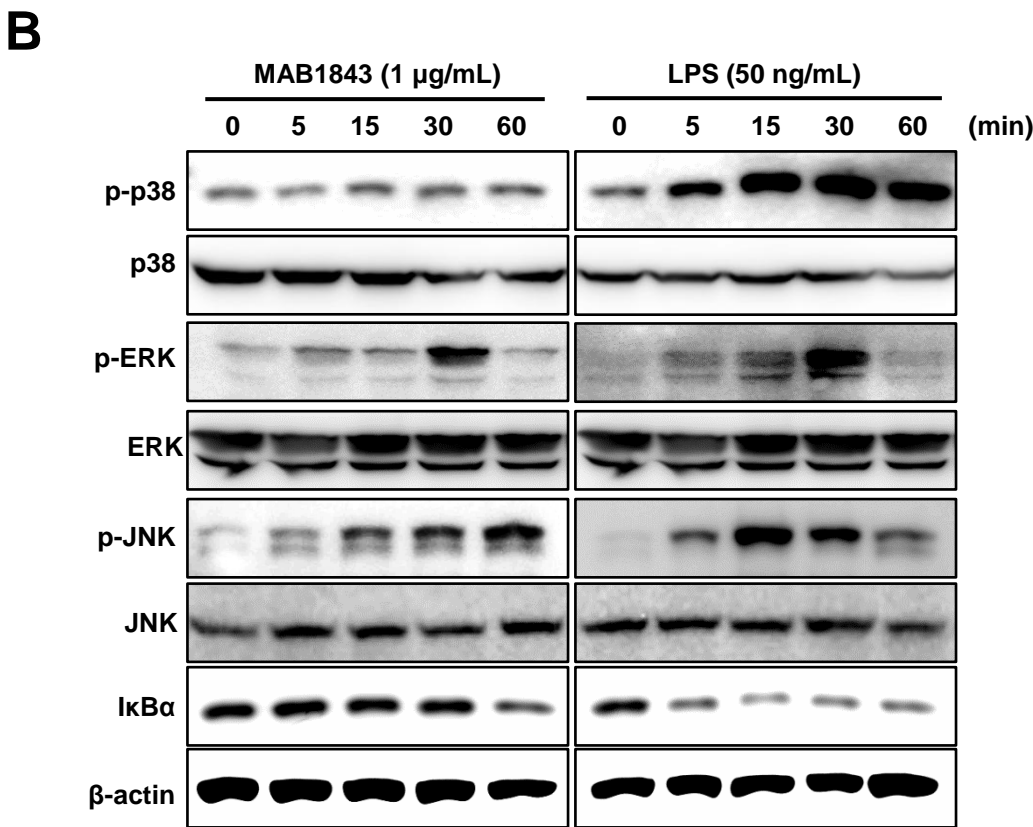
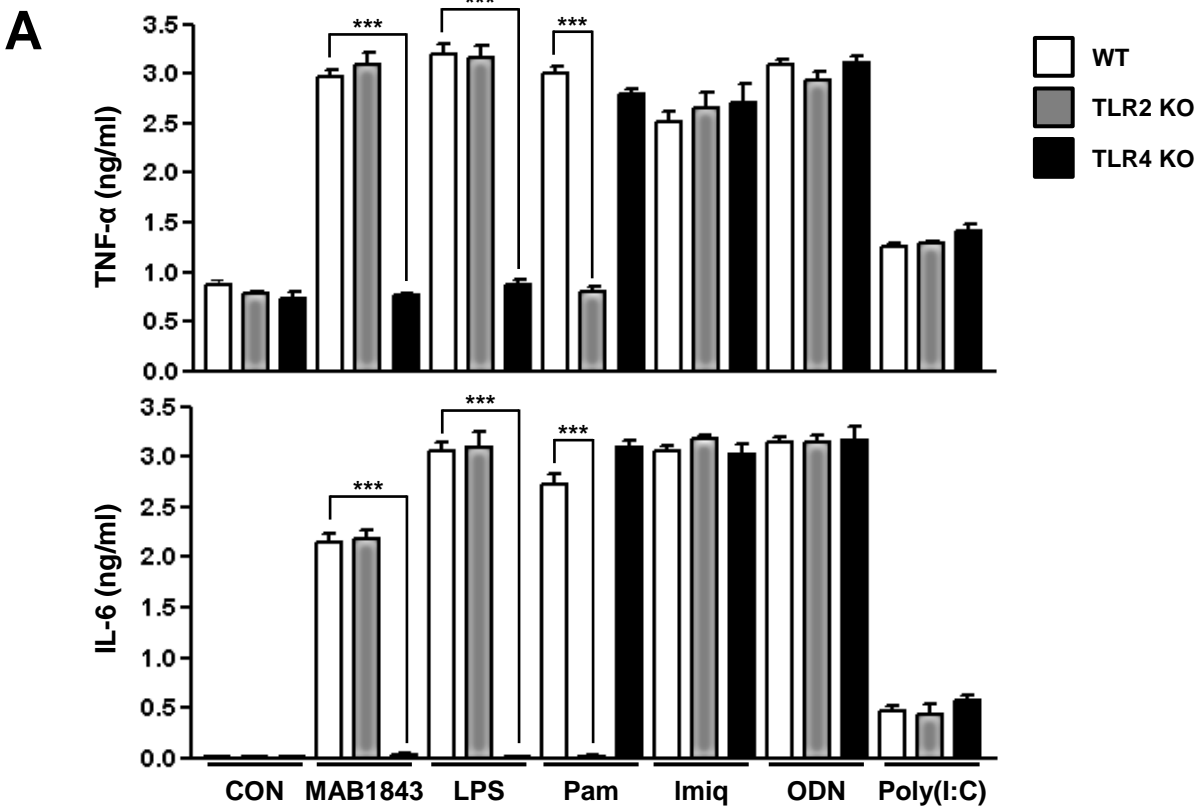
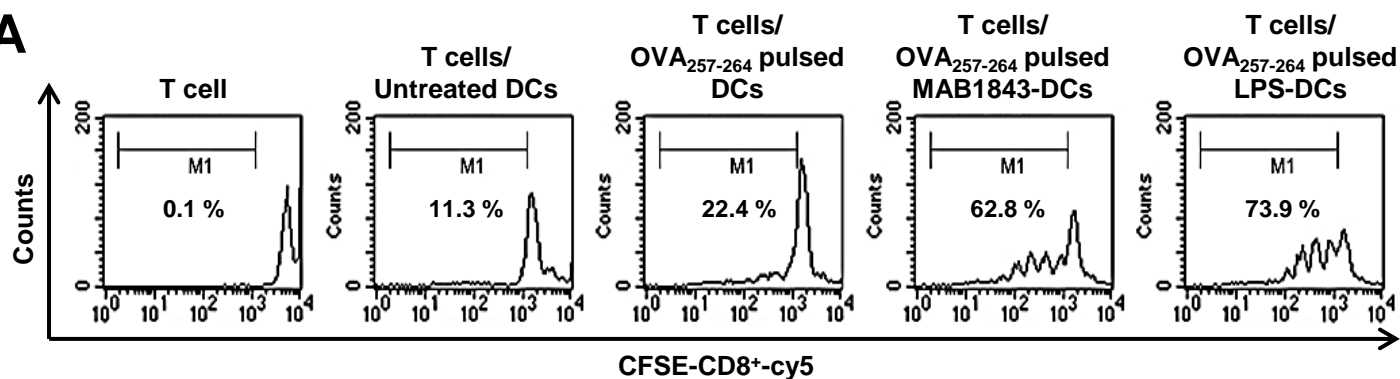
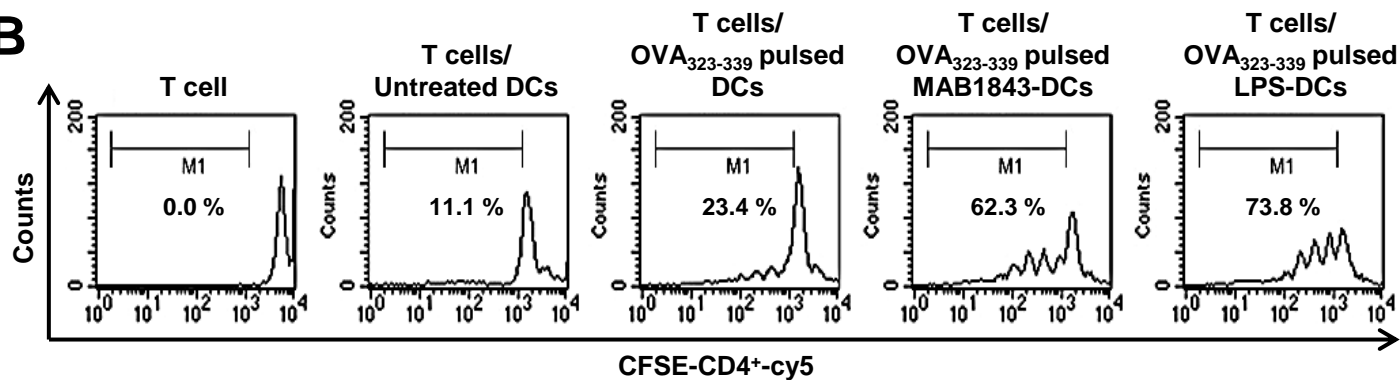


Figure 4

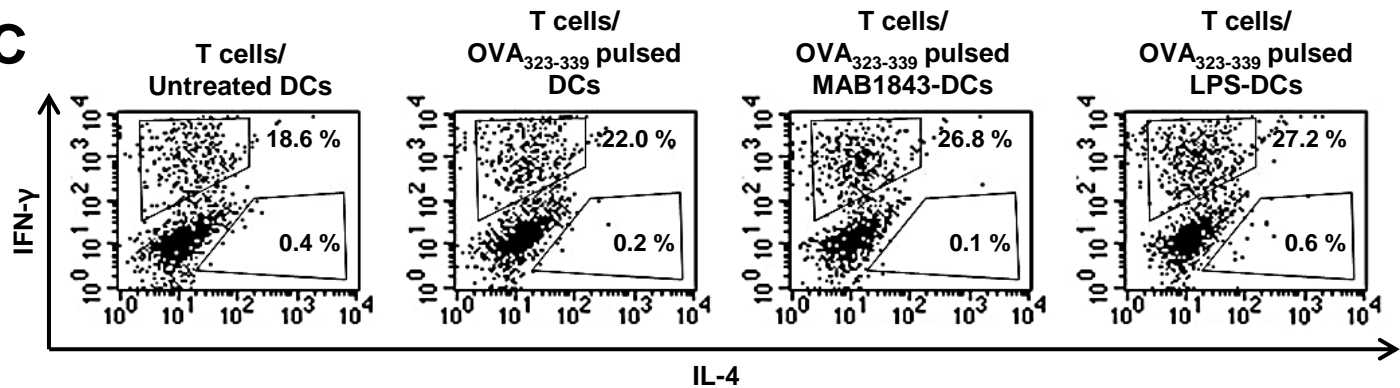
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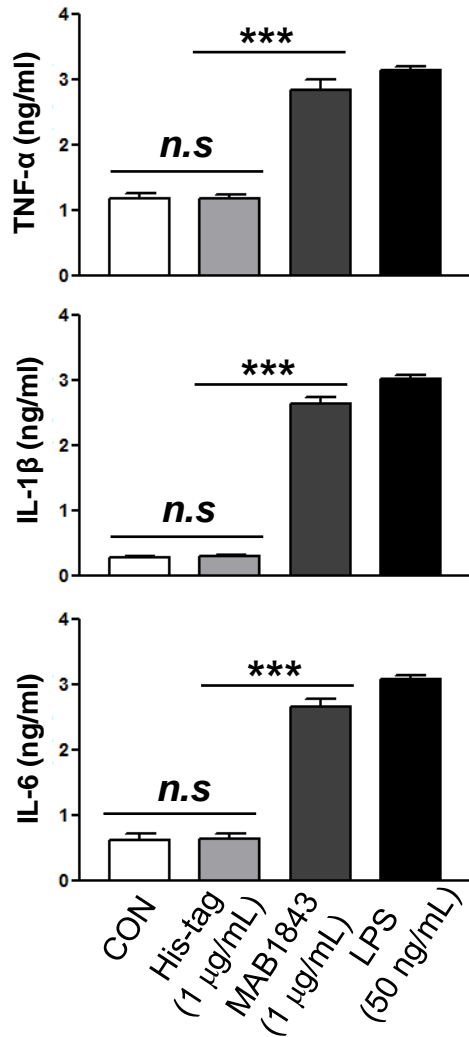
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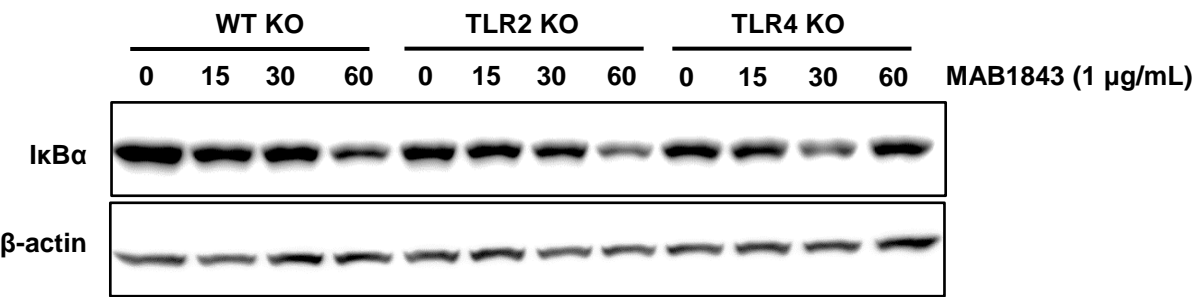


Supplementary Figure 1



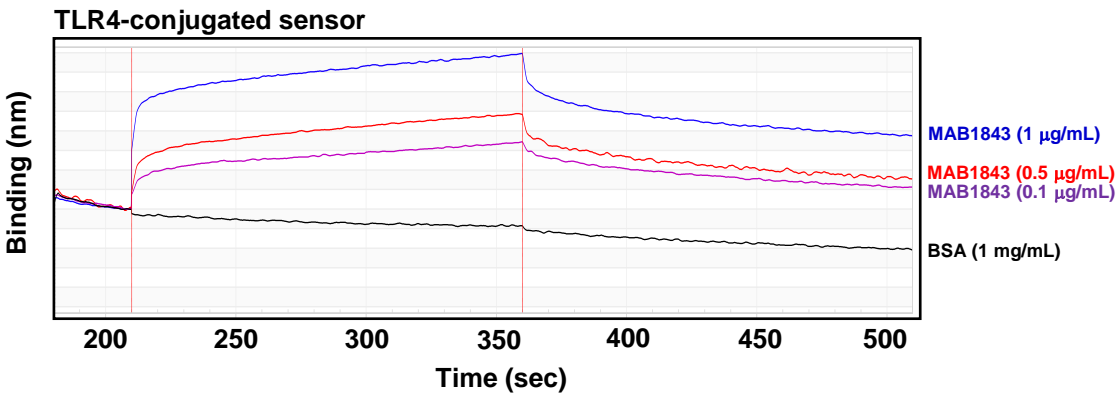
Supplementary Fig. 1. His-tagged protein of MAB1843 does not affect to the cytokine production in DCs. Immature DCs were cultured with 1 μg/mL of His-tag, MAB1843 or 50 ng/mL of LPS for 24 h. The amounts of TNF-α, IL-1β, and IL-6 in the culture medium were measured by ELISA. The experiments were run at least in triplicates. ****P* < 0.001. The results are representative of three independent experiments.

Supplementary Figure 2



Supplementary Fig. 2. MAB1843 induces the activation of $\text{I}\kappa\text{B}$ via TLR4 in DCs. DCs-derived from WT, TLR2 KO, and TLR4 KO mouse were treated with MAB1843 (1 $\mu\text{g/mL}$) for 0, 5, 30, and 60 min. Anti- $\text{I}\kappa\text{B}\alpha$ antibody and anti- β -actin antibody was added to the western blot membrane. The results are representative of three independent experiments.

Supplementary Figure 3



Supplementary Fig. 3. Direct binding of MAB1843 to TLR4. Blitz assay to measure the binding of TLR4 to various doses of MAB1843 or PBS. The vertical and horizontal axes represent the light-shift distance (nm) in the solution and time (sec), respectively.